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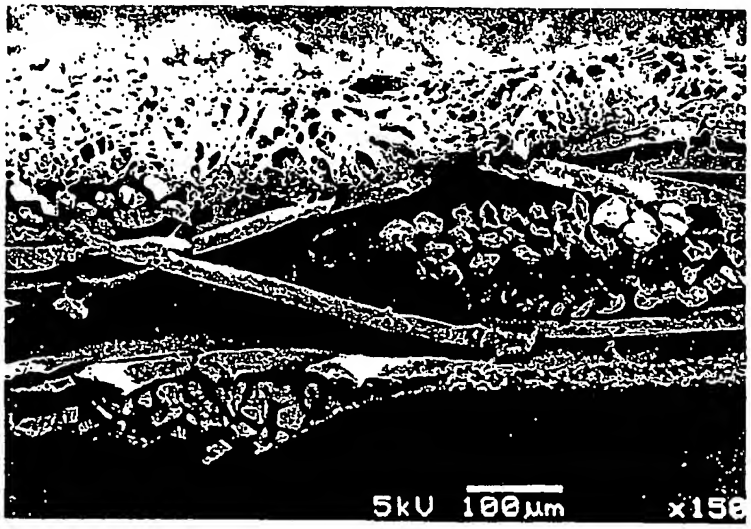
(54) **MATERIALS FOR CULTURING CARDIOVASCULAR TISSUES AND METHOD OF TISSUTE REGENERATION**

(57) Materials for culturing cardiovascular tissues wherein a sponge made of a bioabsorbable material is reinforced with a reinforcement made of a bioabsorbable material.

F I G . 1



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Description

TECHNICAL FIELD

[0001] The present invention relates to a matrix for culturing cardiovascular cells to regenerate cardiovascular tissue and a method for regenerating cardiovascular tissue such as an artificial blood vessel, cardiac valve, pericardium, etc.

BACKGROUND ART

[0002] In the field of artificial vessels, for instance, those made of non-bioabsorbable polymers are widely used. An artificial vessel (GORE-TEX), for example, is used most frequently in a clinical field. Such non-bioabsorbable artificial vessel is excellent in physical properties; however, because of the non-bioabsorbability, it remains in vivo as a foreign body for a long period of time after implantation. Further, when the non-bioabsorbable artificial vessel is implanted into the body of a child, another surgery for replacement is necessary since the non-bioabsorbable artificial vessel does not expand with the growth of the autogeneous blood vessel.

[0003] A tissue regeneration method employing tissue engineering techniques has recently been developed, wherein cells of autogeneous tissue are seeded and cultured on a scaffold made of a bioabsorbable polymer so as to regenerate the autogeneous tissue. There have been published quite a few research reports of the tissue regeneration method applied to skin regeneration (M. L. Cooper, L. F. Hansbrough, R. L. Spielvogel, et al.: In vivo optimization of a living dermal substitute employing cultured human fibroblasts on a biodegradable polyglycolic acid or polyglactin mesh. Biomaterials, 12: 243-248, 1991) and cartilage regeneration (C.A. Vacanti, R. Langer, et al.: Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation. Plast. Reconstr. Surg., 88:753-759, 1991).

[0004] If a blood vessel can be regenerated in the same manner as described above, growth of the regenerated blood vessel is expected since it is regenerated by using autogeneous tissue and no longer necessitates the use of anti-coagulants.

[0005] An object of the present invention is to provide a matrix which allows cells to sufficiently adhere thereto, provides an optimum scaffold for cell proliferation, maintains satisfactory blood flow resistance in vivo till autogeneous tissue is regenerated, and is ultimately decomposed and absorbed in vivo.

BRIEF DESCRIPTION OF DRAWINGS

[0006]

Fig. 1 is a photograph showing a cross-sectional view of a vascular regeneration matrix according to the present invention.

Fig. 2 is a photograph showing a plan view of a vascular regeneration matrix according to the present invention.

Fig. 3 is a photograph of the angiogram recorded at the 3rd postoperative month.

DISCLOSURE OF INVENTION

[0007] Basic requirements for the matrix for culturing cardiovascular cells to regenerate cardiovascular tissue are an ability to allow cells seeded thereon to adhere firmly thereon and a bioabsorbability which enables the matrix to be absorbed in vivo when a blood vessel is regenerated. A sponge is considered to be the optimum material to fulfill the above requirements.

[0008] In the case of using the matrix for regenerating a blood vessel, the matrix is required to maintain an enough strength to endure a blood flow for a certain period of time after implantation till the blood vessel is regenerated in vivo.

[0009] The inventors found that the above object is achieved by strengthening, with a reinforcement made of a bioabsorbable material, a sponge made of a bioabsorbable material which is an optimum scaffold for cell proliferation and excellent in cell adhesiveness.

[0010] The present invention provides a matrix for culturing cardiovascular tissue and a method for regenerating cardiovascular tissue of the following items.

[0011] Item 1. A matrix for culturing cardiovascular cells to regenerate cardiovascular tissue comprising a sponge made of a bioabsorbable material and a reinforcement made of a bioabsorbable material.

[0012] Item 2. The matrix for culturing cardiovascular cells to regenerate cardiovascular tissue according to item 1, wherein the bioabsorbable material is at least one member selected from the group consisting of polyglycolic acid, polylactic acid (D form, L form, DL form), polycaprolactone, glycolic acid-lactic acid (D form, L form, DL form) copolymer, glycolic acid-caprolactone copolymer, lactic acid (D form, L form, DL form)-caprolactone copolymer, poly(p-dioxanone) and the like.

[0013] Item 3. The matrix for culturing cardiovascular cells to regenerate cardiovascular tissue according to item 1 for use in regenerating an artery, wherein the sponge comprises a lactic acid-caprolactone copolymer and the reinforcement comprises polylactic acid.

[0014] Item 4. The matrix for culturing cardiovascular cells to regenerate cardiovascular tissue according to item 1 for use in regenerating a vein, wherein the sponge comprises a lactic acid-caprolactone copolymer and the reinforcement comprises polyglycolic acid.

[0015] Item 5. The matrix for culturing cardiovascular cells to regenerate cardiovascular tissue according to item 1 for use in regenerating a cardiac valve or a pericardium, wherein the sponge comprises a lactic acid-caprolactone copolymer and the reinforcement comprises polylactic acid.

[0016] Item 6. The matrix for culturing cardiovascular

cells to regenerate cardiovascular tissue according to item 1, wherein the sponge has a pore diameter of about 5 μm to about 100 μm.

[0017] Item 7. A method for regenerating cardiovascular tissue comprising seeding cells on the matrix of item 1 and culturing the cells.

[0018] Item 8. The method for regenerating cardiovascular tissue according to item 7, wherein the cardiovascular tissue to be regenerated is a blood vessel.

[0019] Item 9. The method for regenerating cardiovascular tissue according to item 7, wherein the cardiovascular tissue to be regenerated is a cardiac valve.

[0020] Item 10. The method for regenerating cardiovascular tissue according to item 7, wherein the cardiovascular tissue to be regenerated is a pericardium. .

[0021] Item 11. The method for regenerating cardiovascular tissue according to item 7, wherein the cells to be seeded are a mixed cell culture of two or three different kinds selected from the group consisting of endothelial cells, smooth muscle cells and fibroblasts.

[0022] According to the invention, it is preferable that regeneration of cardiovascular tissue be conducted by seeding cells to a matrix for culturing cardiovascular cells and embedding the matrix in vivo to regenerate cardiovascular tissues in vivo.

[0023] Examples of bioabsorbable material include polyglycolic acid, polylactic acid (D form, L form, DL form), polycaprolactone, glycolic acid-lactic acid (D form, L form, DL form) copolymer, glycolic acid-caprolactone copolymer, lactic acid (D form, L form, DL form)-caprolactone copolymer, poly(p-dioxanone) and the like.

[0024] Examples of cardiovascular tissue include blood vessels, cardiac valves, the pericardium and the like.

[0025] The matrix of the invention is obtained by strengthening a sponge made of a bioabsorbable material with a reinforcement (in the form of a fiber, nonwoven fabric or film) made of a bioabsorbable material. There is no limitation on the bio-absorbable materials to be used for the sponge and the reinforcement. In the case of preparing the matrix for regenerating a blood vessel, a sponge made of a lactic acid-caprolactone copolymer may be combined with a reinforcement made of polylactic acid when the blood vessel is an artery, and the same sponge may be combined with a reinforcement made of polyglycolic acid when the blood vessel is a vein. Further, in the case of regenerating a cardiac valve or the pericardium, a sponge made of a lactic acid-caprolactone copolymer may be combined with a reinforcement made of polylactic acid.

[0026] The sponge has pores each having such a pore size that cells can suitably be adhered thereto to proliferate and that no blood leakage is caused when the matrix comprising the sponge is implanted as cardiovascular tissue. The pore size may typically be about 1 mm or less, preferably about 5-100 μm. The shape of the matrix may be cylindrical when the cardiovascular tissue to be regenerated is a blood vessel or may be plane when the cardiovascular tissue to be regenerated is a cardiac valve or the pericardium. In the case of regenerating a blood vessel, the length and inside diameter of the matrix may be adjusted depending on the target blood vessel. The thickness of the matrix is chosen depending on the desired period for bio-absorption or ease of suturing. The thickness may typically be about 5 mm or less, preferably from about 500 μm to about 2 mm.

[0027] For preparation of the sponge, the following alternative processes, among others, are available.

(1) Lyophilization process

[0028] A substrate polymer solution is poured in a mold, frozen, and, then lyophilized. According to the freezing temperature and polymer concentration, sponges having various pore diameters are obtained (described in Examples).

(2) Elution process

[0029] A water-soluble material is mixed with the substrate polymer solution and, after drying, the water-soluble material is washed out with rinse water. The resultant sponge has a pore diameter corresponding to the particle size of the water-soluble material used. In the present case, sucrose can be used with advantage.

[0030] The reinforcement must have a greater strength than the sponge. The reinforcement can be selected from among a fiber, nonwoven cloth, film and so on.

[0031] The reinforcement is preferably integrated with the sponge and can be located either on the interior surface, inside, or exterior surface of the sponge. However, since the interior surface of the sponge is involved in the adhesion of vascular endothelial cells, it is preferably situated inside or on the exterior surface, although the interior surface may be optionally used.

[0032] As to the cells to be seeded, substantially the same kinds of cells are used for various cardiovascular tissues in common. Thus, they are endothelial cells, smooth muscle cells and fibroblasts, and a mixed cell culture of two or three different kinds of cells can be mentioned by way of example. Tissue construction is carried out using such mixed culture cells.

[0033] The cultural conditions for the cells to be used and the seeding method are described below.

A. Cell isolation, culture, and propagation

[0034] The vascular tissue isolated in a sterile environment is immersed in a cell culture medium and washed with phosphate-buffered saline in a clean bench. Then, on a Petri dish, the tissue is cut into pieces using a surgical knife according to the simple explant technique. Tissue pieces sized about 1-2 mm^2 are dis-

tributed uniformly on the dish and after about 20 minutes, when the tissue pieces have intimately adhered to the bottom of the dish, a culture medium is added. As the culture medium, Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10 % fetal calf serum and 1 % antibiotics solution (L-glutamine 29.2 mg/ml, penicillin G 1000 U/ml, streptomycin sulfate 10,000 μg/ml) is used. The mixed cells of endothelial cells and fibroblasts begin to migrate from the tissue pieces on the dish after 5-7 days, forming mixed-cell colonies around the explants in a further one week. After another 2-3 weeks, the mixed cells become confluent on the dish. Immediately, a passage is made using 0.25 % trypsin and the culture in a 75 cm^2 culture flask is started. Generally when the growth in this flask has become confluent, about $2x10^6$ cells are available. Cell culture is performed under an atmosphere comprising 5 % CO_2 and 21 % O_2 and continued until $10x10^6$ cells have been obtained. While the culture medium is renewed every 4-5 days, the resultant of a preliminary experiment has shown that the doubling time of cells is about 48 hours. Incidentally, the counting of cell population during the course is carried out by the classical exclusion method using Trypan Blue.

B. Cell sorting and endothelial cell purification

[0035] At the stage when the mixed cells have become confluent and a reasonable number of cells is obtained, endothelial cells are sorted out from among the mixed cells using FACS according to the following protocol. Thus, Dil-acetylated LDL (fluorescent dye marker; product of Biomedical Technologies) (briefly, Dil-Ac-LDL) is added to the mixed cell culture at a concentration of 1 μg/ml, followed by 24-hour incubation. This marker is taken up intracellularly through a scavenger pathway specific to endothelial cells and macrophages. After 24 hours, the cells are trypsinized to prepare a mixed cell suspension and sorting is performed using a cell sorter (FACS machine; product of Bectin Dickenson). According to the size and emission of fluorescence, the cells are sorted into Dil-Ac-LDL-positive cells and Dil-Ac-LDL-negative cells. After the sorting, these types of cells are independently cultured and the culture is continued until $2x10^6$ endothelial cells are obtained.

C. Tissue construction

[0036] The first step of tissue construction comprises seeding cells in vitro. Specifically, a biodegradable culture matrix is seeded with about $1x10^6$ cells/cm^2 of Dil-Ac-LDL-negative fibroblasts.

[0037] Immediately following the seeding of a concentrated cell suspension on the matrix, the system is allowed to stand on the culture dish in a clean bench for 30-60 minutes, and thereafter about 50 ml of a culture medium is added. The culture medium is renewed every day as a rule and after 7 days, that is, one day before surgical transplantation, a further seeding is performed with a suspension of endothelial cells (about $2x10^6$ cells), whereby a monolayer of endothelial cells is obtained.

[0038] The above steps A-C show the cell isolation, culture and seeding procedures for the construction of a heart valve, a pericardium, or a blood vessel.

BEST MODE FOR CARRYING OUT THE INVENTION

[0039] The following examples are further illustrative of the present invention.

Example 1

· Construction of a vascular regeneration matrix

[0040] A glass test tube (10 mm in outside diameter) was wrapped around with a plain-weave cloth of poly-L-lactide fiber (photograph) in a double-cylindrical form. This assembly was set in a mold (12 mm in inside diameter) and a solution of L-lactide-caprolactone copolymer (50:50) in dioxane (5 %) was poured into the clearance, frozen and then lyophilized.

[0041] The cylindrical vascular prosthesis thus obtained was a cellular substrate reinforced with a fibrous material (Figs. 1 and 2).

· Cell culture

[0042] Through a small skin incision, a peripheral vein segment, about 5 mm long, was excised in a sterile environment and immediately immersed in the tissue culture medium. Cell isolation was carried out by the simple explant technique. As the cell culture medium, the standard cell culture medium DMEM mentioned above was used, and the medium was renewed every 2-3 days.

· Seeding of cells

[0043] The matrix prepared above was seeded with about $1x10^6$ cells/cm^2 of a mixed culture of endothelial cells and fibroblasts and the culture was continued for about 1 week until the matrix surface had been completely covered with the cells.

· Animal experiment

[0044] The vascular prosthesis constructed as above was transplanted in the inferior vena cava of a young dog. As a result, no obliteration by rupture was found and a good patency could be verified angiographically at the 3rd postoperative month (the angiograph in Fig. 3). Thoracotomy at 6 months revealed regeneration of the autogenous blood vessel in agreement with the transplantation site.

[0045] In contrast, the matrix not reinforced with poly-

L-lactide fiber ruptured in one week after substitution and the experimental animal succumbed to sudden death.

Claims

1. A matrix for culturing cardiovascular cells to regenerate cardiovascular tissue comprising a sponge made of a bioabsorbable material and a reinforcement made of a bioabsorbable material.

2. The matrix for culturing cardiovascular cells to regenerate cardiovascular tissue according to Claim 1, wherein the bioabsorbable material is at least one member selected from the group consisting of polyglycolic acid, polylactic acid (D form, L form, DL form), polycaprolactone, glycolic acid-lactic acid (D form, L form, DL form) copolymer, glycolic acid-caprolactone copolymer, lactic acid (D form, L form, DL form)-caprolactone copolymer and poly(p-dioxanone).

3. The matrix for culturing cardiovascular cells to regenerate cardiovascular tissue according to Claim 1 for use in regenerating an artery, wherein the sponge comprises a lactic acid-caprolactone copolymer and the reinforcement comprises polylactic acid.

4. The matrix for culturing cardiovascular cells to regenerate cardiovascular tissue according to Claim 1 for use in regenerating a vein, wherein the sponge comprises a lactic acid-caprolactone copolymer and the reinforcement comprises polyglycolic acid.

5. The matrix for culturing cardiovascular cells to regenerate cardiovascular tissue according to Claim 1 for use in regenerating a cardiac valve or a pericardium, wherein the sponge comprises a lactic acid-caprolactone copolymer and the reinforcement comprises polylactic acid.

6. The matrix for culturing cardiovascular cells to regenerate cardiovascular tissue according to Claim 1, wherein the sponge has a pore diameter of about 5 μm to about 100 μm.

7. A method for regenerating cardiovascular tissue comprising seeding cells on the matrix of Claim 1 and culturing the cells.

8. The method for regenerating cardiovascular tissue according to Claim 7, wherein the cardiovascular tissue to be regenerated is a blood vessel.

9. The method for regenerating cardiovascular tissue according to Claim 7, wherein the cardiovascular tissue to be regenerated is a cardiac valve.

10. The method for regenerating cardiovascular tissue according to Claim 7, wherein the cardiovascular tissue to be regenerated is a pericardium.

11. The method for regenerating cardiovascular tissue according to Claim 7, wherein the cells to be seeded are a mixed cell culture of two or three different kinds selected from the group consisting of endothelial cells, smooth muscle cells and fibroblasts.

FIG. 1

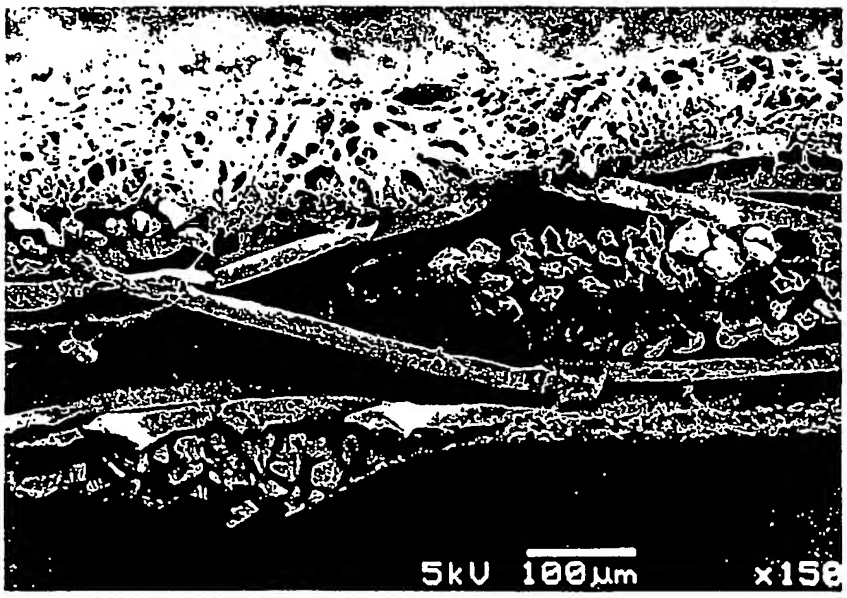

FIG. 2

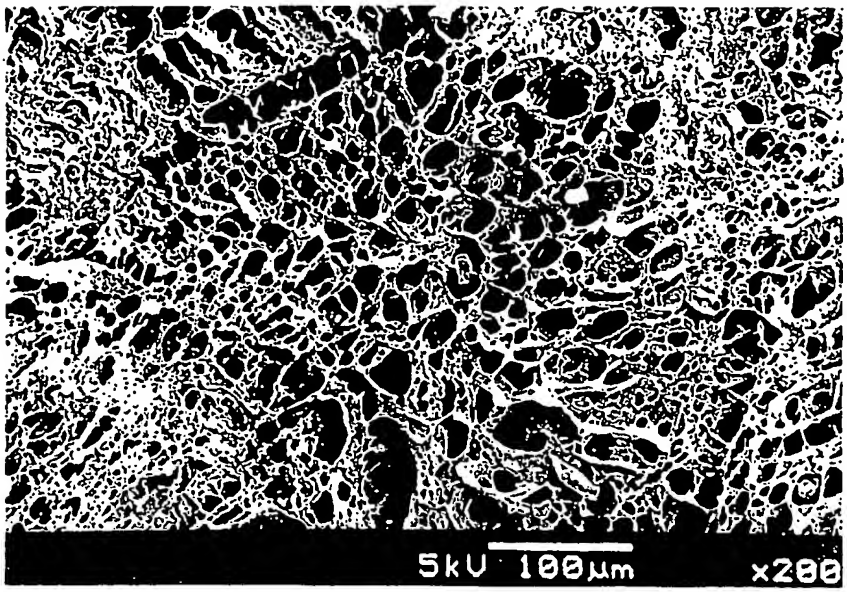

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/06129

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] A61L 27/48, 27/56, 27/58, C12N 5/06, 5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] A61L 27/00-27/60, 33/00-33/18, C12N 5/00-5/28, 11/00-11/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA(STN), REGISTRY(STN), MEDLINE(STN), WPI(DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, 277678, A1 (Stichting Science Park Groningen), 10 August, 1988 (10.08.88), entire description, especially, Claims 1, 2, 6; Column 1; lines 1 to 4; the same column, line 45 to Column 2; line 52; examples 1 to 3, (Example I-III) & JP, 63-272355, A & AU, 8810376, A & NL, 8700113, E & NO, 8800193, A & DK, 8800209, A & FI, 8800147, A	1-6
Y	WO, 96/38188, A1 (Massachusetts Institute of Technology), 05 December, 1996 (05.12.96), entire description, especially, Claims 1, 3, 5; page 7; line 1 to page 8; line 24 & JP, 11-506364, A & US, 5766584, A & EP, 850073, A1 & AU, 9662928, A	1-6
Y	JP, 10-234844, A (Gunze Limited), 08 September, 1998 (08.09.98), entire description, especially, Claims 1, 2, 6; Column 2; lines 27 to 37; Column 3; lines 23 to 31; example 1 (Family: none)	1-6

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November, 2000 (15.11.00)	28 November, 2000 (28.11.00)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/06129

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, 734736, A1 (Toyo Boseki K.K.), 02 October, 1996 (02.10.96), entire description, especially, page 5; lines 38 to 44; example, (Examples) & JP, 8-266613, A & CA, 2173508, A & US, 5723010, A & US, 5876451, A	1-5
A	JP, 5-269196, A (Jinkou Kekkan Gijutsu Kenkyu Center K.K.), 19 October, 1993 (19.10.93), entire description (Family: none)	1-6
A	JP, 5-76588, A (Jinkou Kekkan Gijutsu Kenkyu Center K.K.), 30 March, 1993 (30.03.93), entire description (Family: none)	1-6
A	WO, 84/00302, A1 (Rijk-Suniversiteit te Groningen), 02 February, 1984 (02.02.84), entire description & JP, 59-501300, A & EP, 118458, A1 & NL, 8202893, A & AU, 8317100, A & NO, 8401008, A & BR, 8307439, A & BR, 8307440, A & FI, 8401050, A & DK, 8401067, A & US, 4661530, A & DE, 3374116, G	1-6

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/06129

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 7-11
because they relate to subject matter not required to be searched by this Authority, namely:

They pertain to methods for treatment of the human body by surgery or therapy.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)